Regulation of rat hepatic 3α-hydroxysteroid dehydrogenase in vivo and in primary cultures of rat hepatocytes

R. Todd Stravitz,* Z. Reno Vlahcevic,* W. Michael Pandak,* Andrew Stolz,§ and Phillip B. Hylemon[1,†]

Departments of Medicine* and Microbiology,† Medical College of Virginia, Richmond, VA 23298; and Division of Gastrointestinal and Liver Diseases,§ University of Southern California, Los Angeles, CA 90033

Abstract In the bile acid biosynthetic pathways of humans and the rat, hepatic 3α-hydroxysteroid dehydrogenase (3α-HSDH) catalyzes the stereospecific reduction of the 3-oxo group of bile acid precursors. In addition, 3α-HSDH may serve to shuttle bile acids from sinusoidal to apical (cannalicular) membranes of the rat hepatocyte. The objective of the present study was to define the molecular regulation of rat hepatic 3α-HSDH in response to the key effectors of cholesterol 7α-hydroxylase, the rate-determining enzyme in bile acid biosynthesis. Steady-state 3α-HSDH mRNA levels in primary cultures of rat hepatocytes fell to 16 ± 1% of whole liver levels after 72 h in culture, indicating that the gene is not spontaneously expressed in isolated hepatocytes. However, the addition of thyroxine (1.0 μM) or dexamethasone (1.0 μM) to the culture medium resulted in steady-state mRNA levels of 34 ± 4% and 102 ± 20% of whole liver levels, respectively. Moreover, the combination of thyroxine and dexamethasone (each 1.0 μM) induced mRNA to levels 2-fold higher than whole liver. 3α-HSDH specific activity in cultured hepatocyte cytosol increased from 3.0 ± 0.7 to 10.4 ± 1.3 nmol/min per mg protein in no-addition and thyroxine plus dexamethasone-treated cultures, respectively; protein mass underwent similar changes. Whole liver 3α-HSDH mRNA levels decreased in thyroidectomized, adrenalectomized, and hypophysectomized rats, to 60 ± 6%, 51 ± 4%, and 29 ± 5% of sham-operated rats, respectively. 3α-HSDH transcriptional activity in cultured rat hepatocyte nuclei increased 2.5-fold in response to thyroxine, but was not significantly affected by the addition of dexamethasone, nor by adrenalectomy in vivo. The addition of dexamethasone to hepatocytes treated with actinomycin D prolonged 3α-HSDH mRNA half-life approximately 4-fold over no-addition control cultures. Finally, hydrophobic bile acids and agents known to alter the rate of cholesterol biosynthesis (mevalonate, lovastatin, AY9944) had no effect on steady-state hepatic 3α-HSDH mRNA levels. We conclude that rat hepatic 3α-HSDH gene expression is transcriptionally up-regulated by thyroid hormone and post-transcriptionally up-regulated by glucocorticoids. In contrast to cholesterol 7α-hydroxylase mRNA, 3α-HSDH mRNA is not inhibited by hydrophobic bile acids, or dependent on the supply of newly synthesized cholesterol.—**Stravitz, R. T., Z. R. Vlahcevic, W. M. Pandak, A. Stolz, and P. B. Hylemon.** Regulation of rat hepatic 3α-hydroxysteroid dehydrogenase in vivo and in primary cultures of rat hepatocytes. *J. Lipid Res.* 1994. **35:** 239–247.

Supplementary key words bile acids • dihydrodiol dehydrogenase • bile acid binding protein

Hepatic 3α-hydroxysteroid dehydrogenase (3α-HSDH; EC 1.1.1.50) catalyzes the NAD(P)$^+$-dependent oxidoreduction of many steroids and dihydrodiols, including steroid hormones, prostaglandins, polycyclic aromatic xenobiotics, and cholestanoic acids (1–6). In the bile acid biosynthetic pathways of humans and the rat, hepatic 3α-HSDH catalyzes the 3α-reduction of 7α-hydroxy- and 7α,12α-dihydroxy-5β-cholestane-3-one, metabolic precursors of chenodeoxycholic and cholic acids, respectively (7). In the rat, hepatic 3α-HSDH is also identical to the cytosolic Y' bile acid binding protein, and may be an important determinant of net bile acid transport across the hepatocyte (8–12).

Despite these important and diverse physiologic roles, little is known about the regulation of hepatic 3α-HSDH, and previous studies in the rat have yielded conflicting information. Lax, Ghraf, and Schriefers (13) reported that hepatic 3α-HSDH specific activity decreased in hypophysectomized but not in thyroidectomized, gonadectomized, nor adrenalectomized rats. More recently, 3α-HSDH specific activity in rat liver was induced approximately 50% by the intraperitoneal injection of thyroxine, but was not significantly affected by glucagon, corticosterone, estradiol, or a variety of polycyclic hydrocarbons and bile acid precursors (4). Strong evidence for a role of estrogens in the regulation of hepatic 3α-HSDH activity has been provided by Smithgall and Penning (14), who

Abbreviations: 3α-HSDH, 3α-hydroxysteroid dehydrogenase; T4, L-thyroxine; Dex, dexamethasone; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBST, Tris-buffer saline/Tween 20.

[1]To whom correspondence should be addressed.

demonstrated 2-fold higher specific activity levels in female rat liver compared to male rat liver, a difference abolished by ovariectomy and restored by estradiol administration. Furthermore, a 3-fold higher steady-state level of 3α-HSDH mRNA in female rat livers compared to male rat livers has been observed (15). Collectively, these data suggest that hormones may be important regulators of hepatic 3α-HSDH, although the molecular mechanisms of action and the physiologic significance of these effects have not been determined.

Three groups of investigators have reported the successful cloning and sequencing of the rat hepatic 3α-HSDH gene (15–17). The molecular regulation of 3α-HSDH, a non-rate-limiting enzyme in the bile acid biosynthetic pathway, may now be compared and contrasted to the regulation of cholesterol 7α-hydroxylase, the rate-limiting enzyme in the pathway. In primary cultures of rat hepatocytes, we have recently observed synergistic transcriptional up-regulation of cholesterol 7α-hydroxylase by thyroxine (T4) and dexamethasone (Dex) (18), observations consistent with in vivo data (19, 20). Studies in intact rat models and in primary cultures of rat hepatocytes have also revealed transcriptional repression of cholesterol 7α-hydroxylase by hydrophobic bile acids, and transcriptional stimulation by cholesterol and metabolic precursors of cholesterol (21–23). In the present study, we have examined the effects of hormones, bile acids, and cholesterol on 3α-HSDH mRNA levels in cultured rat hepatocytes and whole rat liver.

EXPERIMENTAL PROCEDURES

Materials

EDTA, BSA, T4, Dex, glucagon, dibutyryl-cAMP, actinomycin D, and 17α-ethinylestradiol were obtained from Sigma. AY9944 (*trans*-N,N'-bis-[(2-chlorophenyl)methyl]-1,4-cyclohexanedimethanamine dihydrochloride), and lovastatin (mevinolin) were generous gifts from Wyeth-Ayerst Laboratories and Merck, Sharp, and Dohme Research Laboratories, respectively. The nick translation kit was obtained from GIBCO-BRL, the Protoblot system from Promega, guanidine thiocyanate from Fluka (Ronkonkoma, NY), and bile acids from Calbiochem. All radioisotopes were purchased from New England Nuclear. All other reagents were of the highest quality commercially available.

Experimental animals

Sham-operated, hypophysectomized, adrenalectomized, thyroidectomized, and adrenalectomized/thyroidectomized male Sprague-Dawley rats were purchased from Charles River (Cambridge, MA). Rats were killed 12 days after surgery. Serum T4 was routinely assayed at time of killing in thyroidectomized and hypophysectomized animals, and serum corticosterone was assayed in adrenalectomized and hypophysectomized animals (performed by Hazelton, Vienna, VA), to document complete ablation of the respective organs.

Preparation of cultured adult rat hepatocytes

Monolayer cultures of primary male rat hepatocytes were prepared as described by Bissel and Guzelian (24) with the modifications recently noted (18). Hepatocytes (3.4×10^6 were added to 60-mm plastic petri dishes coated with rat-tail collagen containing 3 ml Williams' E medium, supplemented with insulin (0.25 U/ml) and penicillin. Culture medium was changed every 24 h. T4 and all steroid hormones were added from the time of plating (0 h) and with each change of medium; bile acids were added at 48 h, and glucagon, dibutyryl-cAMP, and cholesterol synthesis inhibitors (lovastatin and AY9944) were added between 48 and 72 h. Mevalonate (2 mM) was added twice, at 48 and 66 h. Cultures were incubated at 37°C in 5% CO_2. Cells were > 90% viable as determined by trypan blue exclusion. Hepatocytes were harvested 72 h or 96 h after plating (0 h).

RNA preparation

Total RNA was isolated as described by Chirgwin et al. (25) with the modifications recently noted (18).

Northern blot hybridization

Total RNA was size-fractionated by electrophoresis in a 1% agarose/7% formaldehyde gel, and transferred to nitrocellulose membranes by overnight capillary blotting (26). The membrane was baked for 2 h at 80°C in a vacuum oven and prehybridized for 1 h at 65°C in 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1% BSA, and 1 mM EDTA (pH 8.0), as described (27). The entire 2.4 kb 3α-HSDH cDNA (including 42 bp of 5' untranslated region, 966 bp of coding region, and 1.3 kb of 3' untranslated region) was labeled with [α-^{32}P]dCTP by nick translation. Approximately 1.5×10^6 dpm/ml of probe was added to fresh hybridization solution, and the membranes were hybridized overnight at 65°C. Membranes were then washed twice in 40 mM Na phosphate (pH 7.2), 5% SDS, 0.5% BSA, 1 mM EDTA (pH 8.0), and twice in 40 mM Na phosphate, 1% SDS, 1 mM EDTA (pH 8.0), with each wash for 45 min at 65°C. The membrane was air-dried and exposed to film (Kodak XAR) for 8–18 h at −70°C.

To ensure equivalent amounts of total mRNA were probed, rat cyclophilin cDNA was labeled with [α-^{32}P]dCTP and used to probe the same membrane, as previously described (18). No changes in steady-state cyclophilin mRNA were observed under any of the experimental conditions examined.

Dot blot hybridization

Total RNA was diluted in 20 × SSC and 7.4% formaldehyde and applied to a nitrocellulose membrane through a Hybri-Dot Manifold from BRL. Membranes were then processed as described for Northern blots. Quantitation of autoradiograms was performed with a Shimadzu laser densitometer. The ratio of absorbancies for hybridization to 3α-HSDH and cyclophilin were determined for each condition and the resulting indices were compared to control samples.

Nuclear run-on assays

Isolation of nuclei. For cultured hepatocytes, each condition required 40 plates, which were harvested at 72 h. Hepatocytes were scraped into 2 ml PBS per plate with a rubber policeman and pelleted at 2500 rpm for 5 min. Hepatocyte nuclei from whole liver and cultured hepatocytes were isolated as described previously (28).

Transcription assays. RNA labeling and isolation, and the hybridization of nascent mRNA, were performed as previously described (21). The in vitro transcriptional activity of 3α-HSDH was compared to that of rat cyclophilin, which was not altered by the experimental variables (18).

Estimation of 3α-HSDH mRNA half-life

Actinomycin D (10 μg/ml) was added to hepatocyte cultures after overnight incubation with or without Dex (1.0 μM); control cultures were harvested for RNA at the time of actinomycin addition (time 0). This concentration of actinomycin D has been shown to decrease the incorporation of radiolabeled uridine in primary cultures of rat hepatocytes by > 98% (29). Total RNA was harvested from cultures at the times noted after actinomycin addition, subjected to Northern blot hybridization to 3α-HSDH and cyclophilin cDNAs, and quantitated by laser densitometry.

Isolation of hepatocyte cytosol

Hepatocytes (10 plates per condition) were scraped into 2 ml isolation buffer (Tris 50 mM, pH 8.6; sucrose 250 mM; EDTA 1 mM, pH 8.0; 2-mercaptoethanol 1 mM) at 4°C (14) and sonicated, and the lysate was centrifuged at 10,000 *g* for 10 min. The supernatant was then centrifuged at 100,000 *g* for 1.5 h at 4°C. Protein was determined by the method of Bradford (30).

3α-HSDH specific activity

Synthesis of [^{14}C]lithocholate. [24-^{14}C]Lithocholate substrate was synthesized from [24-^{14}C]chenodeoxycholate using a protein extract from *Eubacterium* sp. VPI 12708 (31). In a 10 ml reaction, bacterial protein (15 mg) was incubated with [24-^{14}C]chenodeoxycholate (20 μCi), NAD (50 μM), sodium acetate/MOPS, pH 7.5 (25 mM) for 10–30 min at 37°C and terminated with 0.5 M HCl. Bile acid metabolites were extracted twice in ethyl acetate and purified by preparative TLC in S-1 solvent system (31).

Oxidation of [^{14}C]lithocholate. Hepatocyte cytosolic protein (30–100 μg) was incubated at 37°C in a 2 ml reaction mixture consisting of 0.5 M glycine–NaOH, pH 9.0 (1), 230 μM $NADP^+$, 5.0 μM unlabeled lithocholate, and 15,000 dpm [24-^{14}C]lithocholate. The reaction was terminated with 0.5 M HCl; bile acids were extracted twice in two volumes of ethyl acetate, dried under nitrogen, and spotted onto silica gel 1B plates. Thin-layer chromatography was performed in S-1 solvent system (32), the plates were exposed to XRP film, and the product ([24-^{14}C]-3-oxo-5β-cholanoic acid) was scraped from the TLC plates and quantitated by liquid scintillation spectrometry. 3α-HSDH activity under these conditions was linear with protein concentration between 30 and 100 μg and with time between 0.5 and 2.0 min. Enzyme activity is reported after 0.5-min incubations as nmol/min per mg protein.

Western blot analysis of 3α-HSDH

Cytosolic protein (10 μg per sample) was separated on a 12.5% polyacrylamide denaturing gel according to the method of Laemmli (33). Separated proteins were then transferred to a PVDF membrane in 20% methanol, 0.192 M glycine, 25 mM Tris-HCl, pH 8.3, using a semi-dry blotter apparatus (Bio-Rad, Richmond, CA) for 30 min at 13 volts. The membrane was then incubated in 5% blotto (Carnation Dry Milk) in Tris-buffered saline/Tween-20 (TBST) overnight at 4°C before incubating with a 1:2000 dilution of monospecific polyclonal rabbit antiserum. 3α-HSDH protein was identified with the Amersham ECL chemiluminescent system using a 1:5000 dilution of anti-rabbit IgG horseradish peroxidase-linked $F(ab_2)$ fragments.

Statistical analysis

Results are reported as mean ± SE.

RESULTS

Regulation of steady-state 3α-HSDH mRNA in primary cultures of rat hepatocytes

The data in **Fig. 1** compare 3α-HSDH mRNA levels in primary rat hepatocyte cultures to those in whole rat liver. Steady-state levels of 3α-HSDH mRNA in freshly isolated (0 h) hepatocytes approximated levels found in whole liver (122 ± 21% of whole liver levels). After 72 h in primary culture, however, 3α-HSDH mRNA levels declined to 16 ± 1% of whole liver levels. The addition of T4 (1.0 μM) to the culture medium increased 3α-HSDH mRNA to 34 ± 4% of whole liver levels. Dex (1.0 μM) restored 3α-HSDH mRNA to 102 ± 20% of whole liver levels. Interestingly, the combination of Dex (1.0 μM) and

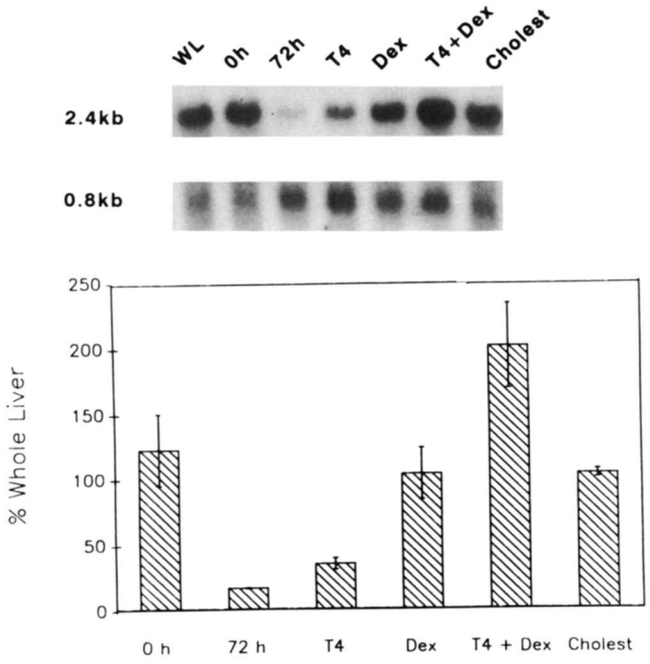

Fig. 1. Steady-state 3α-hydroxysteroid dehydrogenase mRNA levels in primary cultures of rat hepatocytes and whole rat liver. Relative 3α-HSDH mRNA levels in freshly isolated hepatocytes (0 h), 72-h cultured hepatocytes (72 h), 72-h hepatocytes incubated with T4 (1.0 μM), Dex (1.0 μM), T4 + Dex, and in whole liver from cholestyramine-fed rats (5% for 5 days; Cholest). 3α-HSDH is expressed as percentage of levels in whole liver from rats fed a normal diet. 3α-HSDH mRNA was quantitated by dot blot hybridization as described under Experimental Procedures, and was loading-controlled to rat cyclophilin mRNA. (Mean ± SE of three experiments). Top: Northern blot of total RNA from the same cultures. Twenty μg total RNA was separated on a 1% agarose gel, capillary blotted onto nitrocellulose, and hybridized to ^{32}P-labeled 3α-HSDH cDNA (2.4 kb) and cyclophilin cDNA (0.8 kb. WL: whole liver).

T4 (1.0 μM) synergistically increased 3α-HSDH mRNA to 200 ± 32% of whole liver levels. Both L-triiodothyronine and the D stereoisomer of T4 had the same effects as L-T4, which was used in all subsequent studies. Fetal calf serum (10%) also raised 3α-HSDH mRNA an additional 25% over levels in hepatocytes incubated with Dex and T4 alone (data not shown). These effects were specific for 3α-HSDH mRNA, as cyclophilin mRNA levels in 72-h cultures remained unchanged (Fig. 1, Top).

T4 and Dex increased steady-state 3α-HSDH mRNA in a dose-dependent manner. In the experiments shown in **Fig. 2** and **Fig. 3,** cultured hepatocytes were incubated with one hormone to allow adequate detection of the mRNA at 72 h, and the other hormone was added to the culture medium over a wide concentration range. The addition of Dex to hepatocytes incubated with T4 (1.0 μM) yielded maximal steady-state 3α-HSDH mRNA levels at approximately 1.0 μM, with an approximately 8-fold increase above levels in cultures incubated with T4 alone (Fig. 2). An analogous titration for T4 in cultures treated with Dex (0.1 μM) revealed a maximal increase of nearly 2-fold between 0.1 and 1.0 μM (Fig. 3).

To determine whether the T4- and Dex-induced increases in 3α-HSDH mRNA were translated into functional protein, cultured hepatocyte cytosol was subjected to Western immunoblot and determination of 3α-HSDH specific activity. 3α-HSDH immunoreactive protein in 96-h hepatocytes (**Fig. 4**) increased approximately 4-fold in response to Dex (1.0 μM) and 5-fold in response to T4 + Dex (each 1.0 μM), compared to no-addition cultures. 3α-HSDH specific activity, using [24-^{14}C]lithocholate as substrate, underwent increases of similar magnitude (**Fig. 5**). Dex (1.0 μM) alone maintained 3α-HSDH specific activity at the levels of freshly isolated hepatocytes in 72-h cultures (10.1 ± 1.6 and 10.1 ± 1.5 nmol/min per mg protein, respectively), but not in 96-h cultures (6.2 ± 1.6 nmol/min per mg). The administration of T4 (1.0 μM) with Dex was required for continued high levels of 3α-HSDH activity in 96-h cultures (10.4 ± 1.3 nmol/min per mg), an increase of approximately 3.5-fold compared to no-addition cultures. The addition of T4 alone, however, had no detectable effect on 3α-HSDH protein mass or its specific activity.

The evidence that 3α-HSDH specific activity may be stimulated by female sex hormones prompted us to examine the effects of other steroid hormones on 3α-HSDH mRNA levels. The addition of 17α-ethinylestradiol (10 μM), alone and in combination with Dex, T4, and Dex + T4, did not significantly alter 3α-HSDH mRNA (< 10% increase) over levels in hepatocyte cultures incubated without estradiol. Similarly, progesterone (0.1 μM) and testosterone (0.1 μM) added to the culture medium with and without Dex, T4, and Dex + T4 had

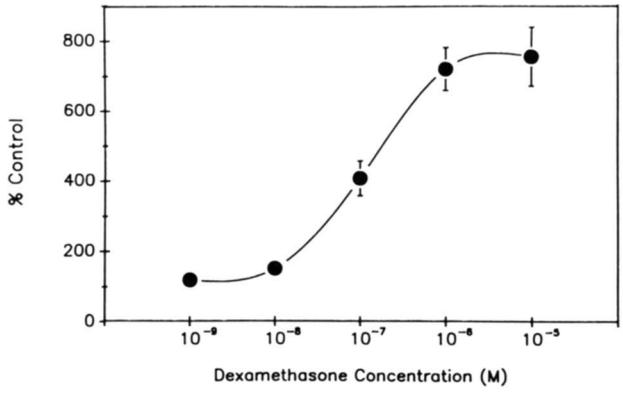

Fig. 2. Effect of dexamethasone concentration on 3α-hydroxysteroid dehydrogenase mRNA levels in primary cultures of rat hepatocytes. Dex in the indicated concentrations was added at 0 h to cultures containing T4 (1.0 μM), and total RNA was harvested at 72 h. 3α-HSDH mRNA levels in Dex-containing cultures were quantitated by dot blot hybridization, loading-controlled to rat cyclophilin mRNA, and expressed as percentage of levels in cultures containing T4 alone (control). (Mean ± SE of three experiments.)

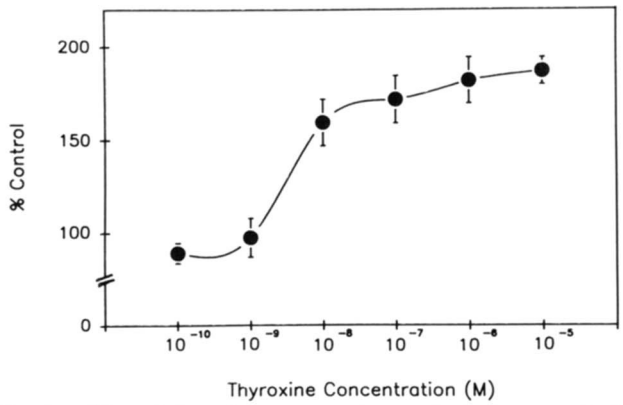

Fig. 3. Effect of thyroxine concentration on 3α-hydroxysteroid dehydrogenase mRNA levels in primary cultures of rat hepatocytes. T4 in the indicated concentrations was added at 0 h to cultures containing Dex (0.1 μM), and total RNA was harvested at 72 h. 3α-HSDH mRNA levels in T4-containing cultures were quantitated by dot blot hybridization, loading-controlled to rat cyclophilin mRNA, and expressed as percentage of levels in cultures containing Dex alone (control). (Mean ± SE of three experiments.)

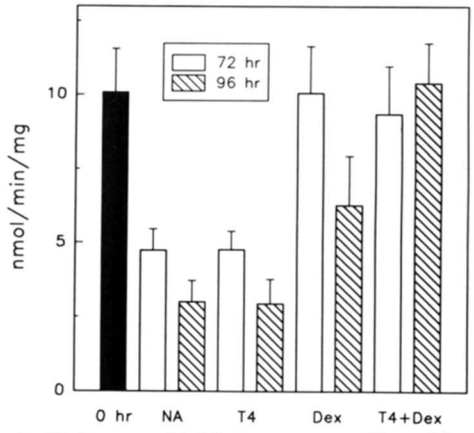

Fig. 5. 3α-Hydroxysteroid dehydrogenase specific activity in fresh rat hepatocyte suspensions and primary cultures of rat hepatocytes. Hepatocyte cytosolic protein (30–100 μg) was incubated for 0.5 min at 37°C in the presence of 230 μM NADP+, 5 μM lithocholate, and 15,000 dpm [24-^{14}C]lithocholate as described in Experimental Procedures. Bile acids were then extracted, resolved by TLC, and the product ([24-^{14}C]-3-oxo-5β-cholanoic acid) was quantitated by liquid scintillation spectrometry. Mean ± SE of n = 6 (0 h), n = 5 (72 h), n = 3 (96 h) experiments.

no effect. Glucagon (0.2 μM), which potently represses cholesterol 7α-hydroxylase mRNA in this model (18), also had no effect (data not shown).

Bile acids and cholesterol availability were also examined as possible regulators of 3α-HSDH gene expression in cultured rat hepatocytes. In the presence of Dex and T4, the addition of bile acids (50 μM) of a wide range in hydrophobicity (tauroursodeoxycholate, taurohyodeoxycholate, taurocholate, taurochenodeoxycholate, and taurodeoxycholate), did not significantly affect 3α-HSDH mRNA levels (decrease of < 10% compared to cultures without added bile acids; n = 2). The cholesterol precursor, mevalonate (2 mM), and inhibitors of cholesterol biosynthesis, lovastatin (20 μM), and AY9944 (20 μM), also had no effect (< 10% change; n = 2) when added to culture medium between 1 and 24 h before harvest (data not shown).

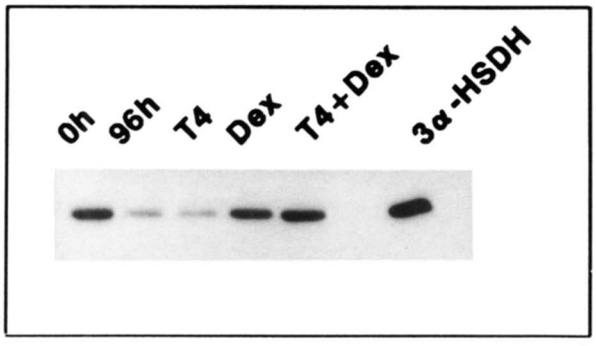

Fig. 4. Western immunoblot of 3α-hydroxysteroid dehydrogenase protein. Cytosolic protein (10 μg) from freshly isolated hepatocyte suspensions (0 h), 96-h cultured hepatocytes from the same rat (96 h), and 96-h hepatocytes incubated with T4 (1.0 μM), Dex (1.0 μM), and T4 + Dex were electrophoresed on a 12.5% polyacrylamide gel, electro-blotted to PVDF membrane, incubated with a monospecific polyclonal rabbit 3α-HSDH rabbit antiserum, and identified with the Amersham ECL system (exposure 20 min) as described in Experimental Procedures. Shown at right, 370 ng of recombinant 3α-HSDH was loaded on the same gel. Laser densitometry of blot (in absorbance units): 0 h, 27,338; 96 h, 6488; T4, 4941; Dex, 25,015; T4 + Dex, 29,921.

Regulation of hepatic 3α-HSDH mRNA in whole rat liver

The physiological significance of thyroid hormone and glucocorticoids in the regulation of 3α-HSDH mRNA was next evaluated in whole rat liver after endocrine organ ablation, the completeness of which was verified by serum thyroid hormone and/or corticosterone levels. Thyroidectomy decreased steady-state levels of 3α-HSDH mRNA in whole rat liver to 60 ± 6% of levels in sham-operated controls (**Fig. 6,** lane A). Similarly,

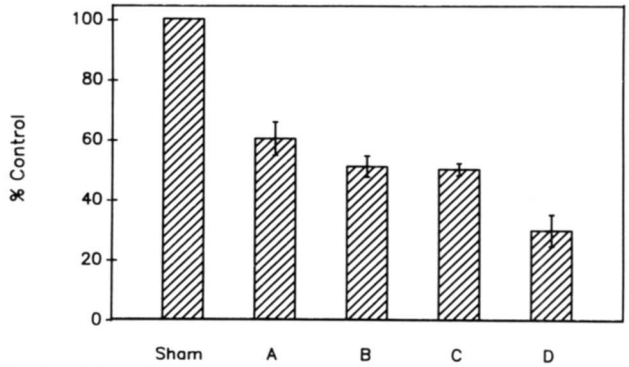

Fig. 6. Whole liver 3α-hydroxysteroid dehydrogenase mRNA levels in sham-operated and endocrine organ-ablated rats. 3α-HSDH mRNA levels from livers of thyroidectomized (A), adrenalectomized (B), thyroidectomized/adrenalectomized (C), and hypophysectomized (D) rats, expressed as percentage of levels in sham-operated controls (Sham). 3α-HSDH mRNA was quantitated by dot blot hybridization and loading-controlled to rat cyclophilin mRNA. (Mean ± SE of three experiments.)

bilateral adrenalectomy (lane B) and combination adrenalectomy/thyroidectomy (lane C) decreased mRNA levels to 51 ± 4% and 50 ± 2% of control, respectively. Hypophysectomy decreased 3α-HSDH mRNA to 29 ± 5% of sham-operated controls (lane D).

The role of bile acid flux across the liver in vivo, believed to be an important determinant of cholesterol 7α-hydroxylase mRNA and activity (21), was also examined as a possible regulator of 3α-HSDH mRNA. Intact rats were fed bile acids of various hydrophobicity for 14 days; others underwent pharmacologic interruption of the enterohepatic circulation of bile acids by treatment with cholestyramine for 5 days. Hepatic 3α-HSDH mRNA levels in rats fed bile acids (chow supplemented with taurocholate or taurochenodeoxycholate 1% w/w, or taurodeoxycholate 0.25% w/w) did not significantly differ (< 10% decrease; n = 2) from normal chow-fed controls (data not shown). Furthermore, 3α-HSDH mRNA levels in whole liver from rats fed a diet containing cholestyramine (5% w/w) did not differ (< 5% increase; n = 3) from levels in whole liver from rats fed a normal diet (Fig. 1).

Transcriptional activity of 3α-HSDH in primary cultures of rat hepatocytes and whole rat liver

To identify the molecular site at which T4 and Dex increase 3α-HSDH mRNA, we determined 3α-HSDH transcriptional activity in primary cultures of rat hepatocytes by a nuclear "run-on" assay (**Fig. 7**). The addition of Dex (0.1 μM) alone had no effect on 3α-HSDH transcriptional activity (mean 106% of no-addition controls). T4 (1.0 μM) and T4 + Dex, however, increased 3α-HSDH transcription approximately 2.5-fold and 3.5-fold, respectively, compared to hepatocytes incubated without either hormone. Hepatic 3α-HSDH transcriptional activity in adrenalectomized rats was also determined to confirm the absence of transcriptional regulation by glucocorticoids. The transcriptional activity of the gene in adrenalectomized rats did not significantly differ from levels in sham-operated controls (mean 87% of control; n = 2).

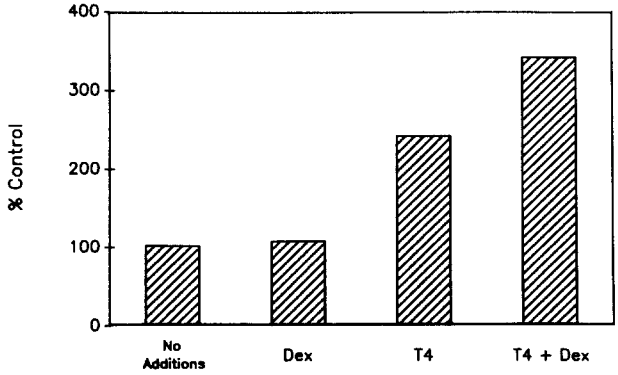

Fig. 7. 3α-Hydroxysteroid dehydrogenase transcriptional activity in primary cultures of rat hepatocytes. Dexamethasone (Dex; 0.1 μM), thyroxine (T4; 1.0 μM), or Dex + T4 were added to hepatocyte cultures at 0 h, and nuclei were harvested at 72 h. Nascent 3α-HSDH and cyclophilin mRNAs were labeled in vitro with [^{32}P]GTP, and hybridized to the corresponding cDNA on nitrocellulose membranes. Membranes were then exposed to X-ray film and quantitated by laser densitometry. The ratio of the absorbancies of 3α-HSDH to cyclophilin was determined for each sample. 3α-HSDH transcriptional activity is expressed as the percentage of these ratios in hormone-treated cultures compared to no-addition control cultures. (Mean of two experiments.)

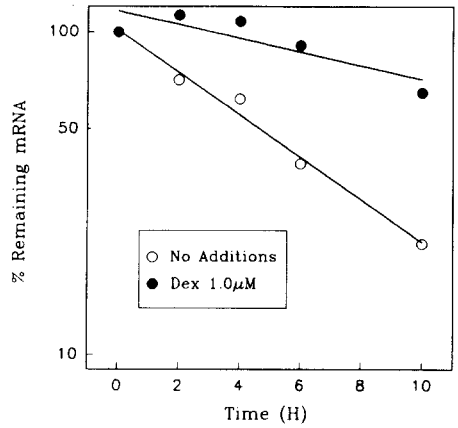

Fig. 8. 3α-Hydroxysteroid dehydrogenase mRNA decay in primary cultures of rat hepatocytes. Semilogarithmic plot of 3α-HSDH mRNA levels, quantitated by Northern blot hybridization, in cultures treated with or without Dex (1.0 μM) at times after the addition of actinomycin D (10 μg/ml). Data are expressed as percentage of control cultures incubated overnight with or without Dex, harvested at the time of actinomycin addition (time 0). For 3α-HSDH mRNA decay in no-addition cultures, r = 0.99, $T_{1/2}$ = 4.7 h. For mRNA decay in Dex-treated cultures, r = 0.89, $T_{1/2}$ by extrapolation = 17.3 h.

Estimation of 3α-HSDH mRNA half-life in hepatocyte cultures

To determine whether Dex increases 3α-HSDH mRNA in vitro by decreasing its rate of degradation, 3α-HSDH mRNA levels in Dex-treated hepatocyte cultures were compared to levels in no-addition cultures after the administration of actinomycin D (**Fig. 8**). Actinomycin D (10 μg/ml) was added (time 0) to hepatocyte cultures incubated overnight with or without Dex (1.0 μM). Total RNA was harvested at regular intervals after the inhibition of transcription, 3α-HSDH mRNA was quantitated by Northern blot hybridization, and the percent of 3α-HSDH mRNA remaining in actinomycin-treated hepatocytes was compared to levels in cells harvested at time 0. Under these conditions, the addition of Dex increased 3α-HSDH mRNA half-life approximately 4-fold (half-life of approximately 4.5 h in the absence and, by extrapolation from Fig. 8, 17 h in the presence of Dex).

DISCUSSION

The purpose of these studies was to assess whether 3α-HSDH, the fourth enzyme in chenodeoxycholate biosynthesis, was coordinately regulated with the first and rate-limiting enzyme in bile acid biosynthesis, cholesterol 7α-hydroxylase. The data of the present study suggest

that thyroid hormone and glucocorticoids may contribute to the coordinate regulation of these two enzymes and possibly to the bile acid biosynthetic pathway as a whole. Studies examining the regulation of other enzymes in the pathway by thyroid hormone and glucocorticoids support this concept. For example, the activity of microsomal 12α-hydroxylase, the cytochrome P450-dependent enzyme largely responsible for determining the cholate/chenodeoxycholate ratio in bile, is decreased in rats treated with thyroid hormone (34, 35) and may be increased by treatment with Dex (35).

In primary cultures of rat hepatocytes, T4 increases the mRNAs of both 3α-HSDH and cholesterol 7α-hydroxylase (18) by stimulating gene transcription. The physiologic significance of thyroid hormone in hepatic 3α-HSDH mRNA expression is supported by the observed 40% lower levels of steady-state mRNA in thyroidectomized rats compared to sham-operated controls. In contrast to thyroid hormone, glucocorticoids appear to regulate the mRNA levels of both 3α-HSDH and cholesterol 7α-hydroxylase, but by different molecular mechanisms. We have previously shown in cultured rat hepatocytes (18) that Dex increased cholesterol 7α-hydroxylase mRNA by increasing transcription of the gene. In contrast, Dex profoundly increased 3α-HSDH mRNA in the same culture model without affecting the rate of gene transcription. The absence of a significant decrease in 3α-HSDH transcriptional activity in response to adrenalectomy, despite 50% lower levels of steady-state mRNA compared to sham-operated controls, supports a post-transcriptional mechanism of this regulation. Specifically, Dex was shown to prolong the stability of 3α-HSDH mRNA by 4-fold, to an estimated half-life of over 17 h. Glucocorticoids have been reported to regulate the expression of other hepatic genes by changing mRNA half-life (36–38).

It should be emphasized that the effects of thyroid hormone and glucocorticoids on 3α-HSDH mRNA are not simply the result of a nonspecific enhancement of cultured cell viability. For example, in the same culture system, the mRNA of sterol 27-hydroxylase, the mitochondrial cytochrome P450 responsible for oxidative side-chain cleavage of bile acid precursors, is minimally affected by Dex and not at all by T4 (R. T. Stravitz, Z. R. Vlahcevic, and P. B. Hyleman, unpublished observations). Furthermore, the addition of Dex to the culture medium had no effect on the half-life of cyclophilin mRNA. Finally, the significance of thyroid hormone and glucocorticoids in the regulation of this gene was corroborated by data obtained in endocrine organ-ablated rat models.

Although T4 and Dex stimulate 3α-HSDH transcription and slow the degradation of its mRNA in vitro, respectively, these effects do not appear to be entirely translated into functional 3α-HSDH protein. Specifically, the combination of the hormones yielded mRNA levels in 72-h cultures 2-fold above, but protein mass and specific activity only slightly (10%) above, levels in freshly isolated hepatocytes. This discrepancy was especially evident for the effects of T4, which only increased 3α-HSDH specific activity when added with Dex. While the explanation for this disparity is speculative, the data imply that 3α-HSDH protein has an extremely long half-life, and therefore steady-state levels may not have been achieved by the time of cell harvest. As shown in Fig. 5, 3α-HSDH specific activity in 72-h no-addition cultures, in which 3α-HSDH mRNA is almost undetectable, declined to approximately 50% of 0 h levels, suggesting a protein half-life as long as 72 h. It should be noted that the sensitivity and specificity of the 3α-HSDH assay developed for these experiments should have distinguished small changes in enzyme activity, and represents a significant improvement over the spectrophotometric assay used in previous studies (1).

Our data suggest that hormones other than thyroxine and glucocorticoids may also up-regulate hepatic 3α-HSDH mRNA. Specifically, the addition of 10% fetal calf serum to hepatocyte cultures incubated with T4 and Dex increased 3α-HSDH mRNA by approximately 25% above the levels in T4 + Dex-treated cultures, and hypophysectomy decreased 3α-HSDH mRNA levels to a greater extent than thyroidectomy/adrenalectomy. Considerable evidence supports a role for estrogens (14, 15, 39) and possibly growth hormone (39) in the regulation of hepatic 3α-HSDH. We were unable to document a direct effect of estrogens on hepatic 3α-HSDH mRNA in cultured rat hepatocytes, despite the use of high doses (10 μM) of 17α-ethinylestradiol, which is resistant to metabolism by hepatocytes (40). The maintenance of hepatic estrogen receptor concentrations in hypophysectomized rats, however, has been shown to be dependent upon the combination of glucocorticoids and growth hormone (41). The absence of a pituitary hormone, therefore, may underlie the lack of response of 3α-HSDH mRNA to ethinylestradiol in cultures containing Dex. Although female hormones are likely regulators of 3α-HSDH activity and mRNA in the intact rat, our data suggest that these effects are dependent upon the intact hypothalamic–pituitary axis.

Hepatic 3α-HSDH mRNA is not affected by a number of other regulators of cholesterol 7α-hydroxylase mRNA. Glucagon and dibutyryl-cAMP, which transcriptionally repressed cholesterol 7α-hydroxylase mRNA by 80–90% within 6 h in cultured rat hepatocytes (18), had no detectable effect on 3α-HSDH mRNA even after 24 h. Similarly, 3α-HSDH is not subject to feedback repression by hydrophobic bile acids, which transcriptionally decrease cholesterol 7α-hydroxylase mRNA when intestinally infused in rats (21) or when added to the medium of cultured rat hepatocytes (23). The pharmacologic interruption of the enterohepatic circulation of bile acids by

cholestyramine feeding, which increases the mRNA of cholesterol 7α-hydroxylase 4- to 5-fold in the rat (18), also had no effect on steady-state 3α-HSDH mRNA levels. Finally, the availability of newly synthesized cholesterol, which is increased by mevalonate and decreased by cholesterol synthesis inhibitors, also does not regulate levels of 3α-HSDH mRNA. In contrast, mevalonate (2 mM) increased cholesterol 7α-hydroxylase mRNA by 2.5-fold, and lovastatin (20 μM) and AY9944 (20 μM) decreased cholesterol 7α-hydroxylase mRNA by 60% and 90%, respectively, under the same culture conditions (R. T. Stravitz, P. B. Hyleman, and Z. R. Vlahcevic, unpublished observations).

These collective data suggest that rat hepatic 3α-HSDH is regulated by thyroid hormone and glucocorticoids, but not by bile acids or cholesterol. Further investigations will be required to assess whether these hormones also affect the rate of net bile acid transport across the rat hepatocyte.

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REFERENCES

1. Vogel, K., P. Bently, K-L. Platt, and F. Oesch. 1980. Rat liver cytoplasmic dihydrodiol dehydrogenase. *J. Biol. Chem.* **255:** 9621–9625.
2. Penning, T., I. Mukharji, S. Barrows, and P. Talalay. 1984. Purification and properties of a 3α-hydroxysteroid dehydrogenase of rat liver cytosol and its inhibition by anti-inflammatory drugs. *Biochem. J.* **222:** 601–611.
3. Krause, J., and H. Karavolas. 1980. Pituitary 5α-dihydroprogesterone 3α-hydroxysteroid oxidoreductases. Subcellular location and properties of NADH- and NADPH-linked activities. *J. Biol. Chem.* **255:** 11807–11814.
4. Vogel, K., K-L. Platt, P. Petrovic, A. Seidel, and F. Oesch. 1982. Dihydrodiol dehydrogenase: substrate specificity, inducibility, and tissue distribution. *Arch. Toxicol. Suppl.* **5:** 360–364.
5. Penning, T., T. Smithgall, L. Askonas, and R. Sharp. 1986. Rat liver 3α-hydroxysteroid dehydrogenase. *Steroids.* **47:** 221–247.
6. Binstock, J. M., R. B. Iyer, C. V. Hamby, V. A. Fried, I. S. Schwartz, B. I. Weinstein, and A. L. Southren. 1992. Human hepatic 3α-hydroxysteroid dehydrogenase: possible identity with human hepatic chlordecone reductase. *Biochem. Biophys. Res. Commun.* **187:** 760–766.
7. Berseus, O. 1967. Conversion of cholesterol to bile acids in rat: purification and properties of a delta-4-3 ketosteroid 5-beta reductase and a 3-alpha-hydroxysteroid dehydrogenase. *Eur. J. Biochem.* **2:** 493–502.
8. Stolz, A., H. Takikawa, Y. Sugiyama, J. Kuhlenkamp, and N. Kaplowitz. 1987. 3α-Hydroxysteroid dehydrogenase activity of the Y' bile acid binders in rat liver cytosol. *J. Clin. Invest.* **79:** 427–434.
9. Takikawa, H., J. C. Fernandez-Checa, J. Kuhlenkamp, A. Stolz, M. Ookhtens, and N. Kaplowitz. 1991. Effect of indomethacin on the uptake, metabolism and excretion of 3-oxocholic acid: Studies in isolated hepatocytes and perfused rat liver. *Biochim. Biophys. Acta.* **1084:** 247–250.
10. Takikawa, H., M. Ookhtens, A. Stolz, and N. Kaplowitz. 1987. Cyclical oxidation-reduction of the C_3 position on bile acids catalyzed by 3α-hydroxysteroid dehydrogenase. II. Studies in the prograde and retrograde single-pass, perfused rat liver and inhibition by indomethacin. *J. Clin. Invest.* **80:** 861–866.
11. Takikawa, H., A. Stolz, and N. Kaplowitz. 1987. Cyclical oxidation-reduction of the C_3 position on bile acids catalyzed by rat hepatic 3α-hydroxysteroid dehydrogenase. I. Studies with the purified enzyme, isolated rat hepatocytes, and inhibition by indomethacin. *J. Clin. Invest.* **80:** 852–860.
12. Stolz, A., H. Takikawa, M. Ookhtens, and N. Kaplowitz. 1989. The role of cytoplasmic proteins in hepatic bile acid transport. *Annu. Rev. Physiol.* **51:** 161–176.
13. Lax, E., R. Ghraf, and H. Schriefers. 1977. Hormonal regulation of hepatic cytoplasmic 3α-hydroxysteroid dehydrogenase activity in the rat. *Horm. Metab. Res.* **9:** 530–531.
14. Smithgall, T. E., and T. M. Penning. 1985. Sex differences in indomethacin-sensitive 3α-hydroxysteroid dehydrogenase of rat liver cytosol. *Cancer Res.* **45:** 4946–4949.
15. Stolz, A., M. Rahimi-Kiani, D. Ameis, E. Chan, M. Ronk, and J. Shively. 1991. Molecular structure of rat hepatic 3α-hydroxysteroid dehydrogenase—a member of the oxidoreductase gene family. *J. Biol. Chem.* **266:** 15253–15257.
16. Pawlowski, J., M. Huizinga, and T. Penning. 1991. Cloning and sequencing of the cDNA for rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase. *J. Biol. Chem.* **266:** 8820–8825.
17. Cheng, K., P. White, and K. Qin. 1991. Molecular cloning and expression of rat liver 3α-hydroxysteroid dehydrogenase. *Mol. Endocrinol.* **5:** 823–828.
18. Hylemon, P. B., E. C. Gurley, R. T. Stravitz, W. M. Pandak, J. Litz, J. Y. L. Chiang, and Z. R. Vlahcevic. 1992. Hormonal regulation of cholesterol 7α-hydroxylase mRNA levels and transcriptional activity in primary rat hepatocyte cultures. *J. Biol. Chem.* **267:** 16866–16871.
19. Ness, G. C., L. Pendleton, Y. Li, and J. Y. L. Chiang. 1990. Effect of thyroid hormone on hepatic cholesterol 7α-hydroxylase, LDL receptor, HMG-CoA reductase, farnesyl pyrophosphate synthetase, and apolipoprotein A-I mRNA levels in hypophysectomized rats. *Biochem. Biophys. Res. Commun.* **172:** 1150–1156.
20. Van Cantfort, J. 1973. Controle par les glucocorticosteroides de l'activite circadienne de la cholesterol 7α-hydroxylase. *Biochimie.* **55:** 1171–1173.
21. Pandak, W. M., Y. C. Li, J. Y. L. Chiang, E. J. Studer, E. C. Gurley, D. M. Heuman, Z. R. Vlahcevic, and P. B. Hylemon. 1991. Regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by taurocholate and cholesterol in the chronic biliary diverted rat. *J. Biol. Chem.* **266:** 3416–3421.
22. Jones, M. P., W. M. Pandak, D. M. Heuman, J. Y. L. Chiang, P. B. Hylemon, and Z. R. Vlahcevic. 1993. Cholesterol 7α-hydroxylase: evidence for transcriptional regulation by cholesterol or metabolic products of

cholesterol in the rat. *J. Lipid Res.* **34:** 885–892.
23. Stravitz, R. T., P. B. Hylemon, D. M. Heuman, L. R. Hagey, C. D. Schteingart, H-T. Ton-Nu, A. F. Hofmann, and Z. R. Vlahcevic. 1993. Transcriptional regulation of cholesterol 7α-hydroxylase mRNA by conjugated bile acids in primary cultures of rat hepatocytes. *J. Biol. Chem.* **268:** 13987–13993.
24. Bissell, D. M., and P. S. Guzelian. 1980. Phenotypic stability of adult rat hepatocytes in primary monolayer cultures. *Proc. NY Acad. Sci.* **349:** 85–98.
25. Chirgwin, J., A. Przybyla, R. MacDonald, and W. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* **18:** 5294–5299.
26. Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* **77:** 5201–5205.
27. Church, G. M., and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA.* **81:** 1991–1995.
28. Clarke, C., A. Fogelman, and P. Edwards. 1985. Transcriptional regulation of the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene in rat liver. *J. Biol. Chem.* **260:** 14363–14367.
29. Johnson, T., J. Trojan, S. Rudin, B. Blossey, J. Ilan, and J. Ilan. 1991. Effects of actinomycin D and cycloheximide on transcript levels of IGF-I, actin, and albumin in hepatocyte primary cultures treated with growth hormone and insulin. *Mol. Reprod. Dev.* **30:** 95–99.
30. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
31. Hylemon, P. B., P. D. Melone, C. V. Franklund, E. Lund, and I. Björkhem. 1991. Mechanism of intestinal 7α-dehydroxylation of cholic acid: evidence that allo-deoxycholic acid is an inducible side-product. *J. Lipid Res.* **32:** 89–96.
32. Eneroth, P. 1963. Thin-layer chromatography of bile acids. *J. Lipid Res.* **4:** 11–16.
33. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature.* **227:** 680–685.
34. Björkhem, I., H. Danielsson, and J. Gustafsson. 1973. On the effect of thyroid hormone on 26-hydroxylation of C_{27}-steroids in rat liver. *FEBS Lett.* **31:** 20–22.
35. Ishida, H., M. Noshiro, K. Okuda, and M. J. Coon. 1992. Purification and characterization of 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase. *J. Biol. Chem.* **267:** 21319–21323.
36. Simonet, W. S., and G. C. Ness. 1989. Post-transcriptional regulation of 3-hydroxy-3-methylglutaryl-CoA reductase mRNA in rat liver. *J. Biol. Chem.* **264:** 569–573.
37. Simmons, D. L., P. McQuiddy, and C. B. Kasper. 1987. Induction of the hepatic mixed-function oxidase system by synthetic glucocorticoid. *J. Biol. Chem.* **262:** 326–332.
38. Silver, G., L. M. Reid, and K. S. Krauter. 1990. Dexamethasone-mediated regulation of 3-methylcholanthrene-induced cytochrome P-450d mRNA accumulation in primary rat hepatocyte cultures. *J. Biol. Chem.* **265:** 3134–3138.
39. Penning, T., K. Isaacson, and C. Lyttle. 1992. Hormonal regulation of 3α-hydroxysteroid/dihydrodiol dehydrogenase in rat liver cytosol. *Biochem. Pharmacol.* **43:** 1148–1151.
40. Eisenfeld, A. J., and R. F. Aten. 1987. Estrogen receptors and androgen receptors in the mammalian liver. *J. Steroid Biochem.* **27:** 1109–1118.
41. Norstedt, G., O. Wrange, and J-A. Gustafsson. 1981. Multihormonal regulation of the estrogen receptor in rat liver. *Endocrinology.* **108:** 1190–1196.